MICROBIAL CONVERSION OF MILBEMYCINS: HYDROXYLATION OF MILBEMYCIN A_4 AND RELATED COMPOUNDS BY *Cunninghamella echinulata* ATCC 9244

KEIKO NAKAGAWA, SHUNICHI MIYAKOSHI and AKIO TORIKATA

Fermentation Research Laboratories, Sankyo Co., Ltd.,
1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan

KAZUO SATO and YOSHIHISA TSUKAMOTO

Agricultural Chemicals Research Laboratories, Sankyo Co., Ltd.,
1041 Yasu, Yasu-cho, Shiga 520-23, Japan

(Received for publication August 21, 1990)

Many strains of zygomycetes and actinomycetes were found to convert milbemycin A_4 (**1a**) to 13β-hydroxymilbemycin A_4 (**1b**). Among these strains, *Cunninghamella echinulata* ATCC 9244 had the most efficient 13β-hydroxylation ability on milbemycins. In the conversion of milbemycin A_3 (**2a**), 29-hydroxymilbemycin A_4 (**4a**), and 30-hydroxymilbemycin A_4 (**5a**) with this strain, only 13β-hydroxylated products were obtained. On the other hand, starting from milbemycin A_4 (**1a**) and 5-ketomilbemycin A_4 5-oxime (**6a**), 13β,24- and 13β,30-dihydroxy derivatives were also isolated along with 13β-hydroxylated products. Similarly, conversion of milbemycin D (**3a**) and LL-F28249α (**8a**) gave 13β- and 28-hydroxy derivatives (**8b** and **8c**).

Milbemycins are a family of sixteen-membered macrolides produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus*[1~3], and they exhibit potent antiparasitic and pesticidal activities. Similar structural features and biological activities have also been reported for avermectin[4] and LL-F28249[5] isolated from culture broths of *Streptomyces avermitilis* and *Streptomyces cyaneogriseus* subsp. *noncyanogenus*, respectively.

In a previous paper[6], we reported that microbial conversion of milbemycin A_4 (**1a**), for the preparation of new derivatives that could be used as intermediates in new-compound synthesis or as metabolite reference standards in animal metabolism studies, resulted in efficient hydroxylation at the C-30 position of milbemycins and related compounds. During these screening studies we found that a variety of microbial strains converted milbemycin A_4 (**1a**) to the 13β-hydroxy derivative (**1b**). RAMOS TOMBO *et al.* have recently demonstrated microbial conversion of milbemycin derivatives[7]. They described 13β-hydroxylation and 14,15-epoxydation of milbemycins A_3 (**2a**), A_4 (**1a**), and D (**3a**) by the culture of *Streptomyces violascens* ATCC 31560, and 13β-hydroxylation of 5-ketomilbemycin A_4 5-oxime (**6a**) by the microorganism in the presence of dimethyl sulfoxide. This prompted us to describe herein our independent results.

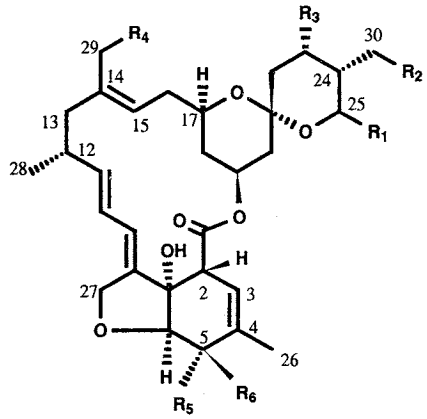
The present paper deals with 13β-hydroxylation of milbemycin A_4 (**1a**) by many strains of zygomycetes and actinomycetes, and with a variety of hydroxylated derivatives of milbemycins and related compounds (Fig. 1) produced by *Cunninghamella echinulata* ATCC 9244 belonging to zygomycetes, the organism that showed the most potent hydroxylation activity on milbemycin A_4 (**1a**) in our study.

Materials and Methods

Materials

Milbemycins A_3 (**2a**)[1], A_4 (**1a**)[1], D (**3a**)[3], 30-hydroxymilbemycin A_4 (**5a**)[6], and LL-F28249α (**8a**)[5] were

Fig. 1. Structures of milbemycins and related compounds.



	R_1	R_2	R_3	R_4	R_5	R_6
1a	CH_2CH_3	H	H	H	H	OH
2a	CH_3	H	H	H	H	OH
3a	$CH(CH_3)_2$	H	H	H	H	OH
4a	CH_2CH_3	H	H	OH	H	OH
5a	CH_2CH_3	OH	H	H	H	OH
6a	CH_2CH_3	H	H	H	NOH	
7a	CH_2CH_3	H	H	H	O	
8a	$C^{31}(CH_3)=CH^{32}CH^{33}(CH_3)_2$	H	OH	H	H	OH

isolated as described previously. 29-Hydroxymilbemycin A_4 (**4a**)[8], 5-ketomilbemycin A_4 5-oxime (**6a**)[9], and 5-ketomilbemycin A_4 (**7a**)[3,9] were prepared from milbemycin A_4 (**1a**) according to literature procedures. 13β,30-Dihydroxymilbemycin A_4 (**1e**), which was isolated as described previously[6], and 13β-hydroxymilbemycins A_3 (**2b**), A_4 (**1b**), and D (**3b**)[10], which were synthesized, were used as authentic reference compounds.

Microorganisms

Microorganisms were obtained from various culture collections and were also isolated from soil samples.

Culture Medium

MY medium consisted of glucose 1.0%, Polypepton (Daigo Nutritive Chemicals) 0.5%, yeast extract (Difco) 0.3%, and malt extract (Difco) 0.3%, pH 6.3~6.5.

Microbial Conversion of Milbemycin A_4 (**1a**)

Each microbial culture was inoculated into 100-ml Erlenmeyer flasks containing 20 ml of MY medium. The flasks were incubated at 200~220 rpm on a rotary shaker for a period of 2 to 3 days at 26 °C for fungi, and at 28 °C for actinomycetes. Then milbemycin A_4 (**1a**) (5% (w/v) in 1,4-dioxane) was added to a final concentration of 500 μg/ml, and cultivation was continued for additional 7 days.

TLC and HPLC Analysis

The culture broths were extracted with EtOAc. The extracts were examined by TLC (Merck Art. No. 5715: EtOAc). Developed chromatograms were detected under 254 nm UV light or by spraying with ammonium molybdate (10% (w/v) in EtOH), followed by warming on a hot plate.

The analytical HPLC was performed using a Nova pak C_{18} (Waters, 8 mm × 10 cm) column. Elution was achieved with one of two solvent systems. System 1 consisted of acetonitrile-water (75:25), with a

flow rate of 1.5 ml/minute. System 2 consisted of acetonitrile-water (55:45), with a flow rate of 1.0 ml/minute. UV-detection was performed at 243 nm.

Isolation of Conversion Products from Milbemycin A_4 (**1a**)

C. echinulata ATCC 9244 was cultured in ten 500-ml Erlenmeyer flasks containing 100 ml of MY medium at 26 °C on a rotary shaker (200~220 rpm). After 3 days cultivation, milbemycin A_4 (**1a**) (5% (w/v) in 1,4-dioxane) was added to a final concentration of 500 μg/ml, and cultivation was continued subsequently for seven additional days. Then the culture broth was filtered and the filtrate was extracted with EtOAc (three times). The mycelium was extracted with 80% MeOH. The MeOH extract was then evaporated and the resulting aqueous solution was extracted with EtOAc (three times). The combined EtOAc extracts were evaporated and chromatographed on silica gel (20~90% EtOAc in *n*-hexane as eluent) to give the 13β-hydroxy derivative (**1b**) and a mixture of two more-polar minor products. Those two minor products were further purified by preparative TLC (Merck Art. No. 5744: EtOAc).

Results and Discussion

Microbial Conversion of Milbemycin A_4 (**1a**) to 13β-Hydroxymilbemycin A_4 (**1b**)

Many strains of zygomycetes and actinomycetes from the type culture collections, and actinomycetes from soil isolation, were found to be capable of converting milbemycin A_4 (**1a**) to the 13β-hydroxy derivative (**1b**). Representative microorganisms that converted milbemycin A_4 (**1a**) to the 13β-hydroxy derivative (**1b**) are shown in Table 1, along with the conversion efficiency determined by the aid of HPLC analysis. According to HPLC analyses of converting products, *C. echinulata* ATCC 9244 seemed to be the most efficient 13β-hydroxylating strain, and it produced two more-polar minor products besides the 13β-hydroxylated product. Therefore, this fungus was employed in the following preparative-scale study for characterizing converted products and was used for subsequent work.

Table 1. Representative microorganisms capable of converting milbemycin A_4 (**1a**) to 13β-hydroxymilbemycin A_4 (**1b**).

Zygomycetes	Conversion efficiency[a]	Actinomycetes	Conversion efficiency
Absidia coerulea IFO 4423	+3	*Amycolata autotrophica* subsp. *canberica* ATCC 35203	+1
A. corymbifera IFO 4009	+1	*Streptomyces acidoresistans* JCM 4713	+2
A. corymbifera IFO 8084	+1	*S. argenteolus* JCM 4623	+1
A. glauca IFO 4003	+2	*S. carbophilus* SANK 62585	+3
Actinomucor elegans ATCC 6476	+1	*S. flavochromogenes* JCM 4752	+1
Cunninghamella echinulata ATCC 9244	+3	*S. jumonjinensis* ATCC 29864	+2
Gongronella butleri IFO 8080	+2	*S. halstedii* NRRL 2138	+1
G. butleri IFO 8081	+1	*S. lavendulae* subsp. *grasserius* JCM 4556	+3
Mortierella vinacea IFO 6738	+1	*S. lipmanii* JCM 4590	+1
Mucor bacilliformis IFO 6414	+1	*S. ornatus* JCM 4502	+3
M. hiemalis IFO 5304	+1	*S. puniceus* JCM 4406	+1
M. hiemalis IFO 5834	+1	*S. purpurascens* JCM 4509	+1
M. hiemalis IFO 6754	+1	*S. roseochromogenes* IFO 3411	+3
M. hiemalis CBS 244.35	+1	*S. roseus* IFO 12818	+1
M. recurvus IFO 8093	+2	*S. spectabillis* JCM 4832	+1
M. odoratus IFO 8637	+1	*S. spiroverticillatus* JCM 4609	+1
Rhizopus chinensis IAM 6013	+1	*S. vinaceus* JCM 4849	+2
R. circinans ATCC 1225	+2	Soil isolate SANK 64587	+3
Zygorhynchus moelleri IFO 4833	+1	Soil isolate SANK 64687	+3

[a] +1: 0.5~10%, +2: 10~30%, +3: more than 30% (HPLC analysis).

Identification of Conversion Products from *C. echinulata* ATCC 9244

The major product and one minor product were identified as 13β-hydroxymilbemycin A_4 (**1b**) and 13β,30-dihydroxymilbemycin A_4 (**1e**), respectively, by comparing IR, MS, and NMR spectra with those of authentic compounds. The second minor product was a new one, which was identified as 13β,24-dihydroxymilbemycin A_4 (**1d**) from its physico-chemical properties.

Application of *C. echinulata* ATCC 9244 for Conversion of Related Compounds

Microbial conversions by *C. echinulata* ATCC 9244 of milbemycin A_3 (**2a**), milbemycin D (**3a**), 29-hydroxymilbemycin A_4 (**4a**), 30-hydroxymilbemycin A_4 (**5a**), 5-ketomilbemycin A_4 5-oxime (**6a**), 5-ketomilbemycin A_4 (**7a**), and LL-F28249α (**8a**) were examined using a similar method as for milbemycin A_4 (**1a**). The results are summarized in Table 2. *C. echinulata* ATCC 9244 was able to convert most of these compounds to corresponding 13β-hydroxy derivatives, and some other hydroxy compounds. The exception was 5-ketomilbemycin A_4 (**7a**). No conversion product from 5-ketomilbemycin A_4 (**7a**) was detected. Therefore, the hydroxyl group of the C-5 position of milbemycin A_4 (**1a**) may be essential for the hydroxylations by *C. echinulata* ATCC 9244. Chromatograms of the hydroxylation products are summarized in Table 3. The spectral evidence in support of the identification of conversion products is

Table 2. Conversion of milbemycins and related compound by *Cunninghamella echinulata* ATCC 9244.

Substrate	Concentration (μg/ml)	Conversion time (days)	Product[a] yield (%)			
			(b)	(c)	(d)	(e)
1a	500	7	26		1.3	0.16
2a	500	8	22			
3a	500	7	5.7	4.4		
4a	290	4	16			
5a	450	4	34			
6a	500	7	18		5.2	1.1
8a	500	5	2.1	6.6		

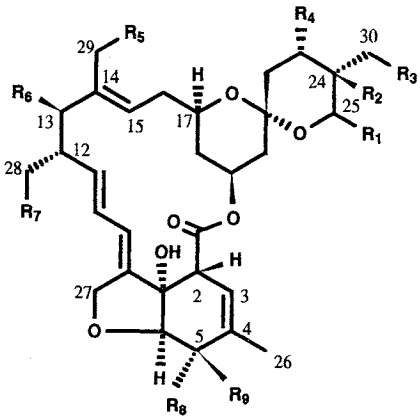
[a] (**b**): 13β-Hydroxy derivative, (**c**): 28-hydroxy derivative, (**d**): 13β,24-dihydroxy derivative, (**e**): 13β,30-dihydroxy derivative.

Table 3. TLC Rf values and HPLC Rt's of milbemycins, related compounds, and conversion products.

Compound[a]	TCL Rf values	HPLC Rt's (minutes)		Compound[a]	TCL Rf values	HPLC Rt's (minutes)	
		System 1	System 2			System 1	System 2
1a	0.59	16.07	—	**4b**	0.18	2.52	5.49
1b	0.46	3.50	10.86	**5a**	0.44	3.08	8.91
1d	0.28	2.06	3.60	**6a**	0.69	18.91	—
1e	0.26	2.00	3.38	**6b**	0.60	3.84	14.75
2a	0.59	11.80	—	**6d**	0.44	2.11	3.94
2b	0.46	3.02	8.04	**6e**	0.39	2.03	3.60
3a	0.62	24.64	—	**7a**	0.68	25.61	—
3b	0.48	4.59	18.26	**8a**	0.55	11.03	—
3c	0.22	6.93	31.97	**8b**	0.42	3.26	10.63
4a	0.38	3.54	11.50	**8c**	0.19	3.89	14.11

[a] **a**: substrate, **b**~**e**: products.

Fig. 2. Structures of conversion products.



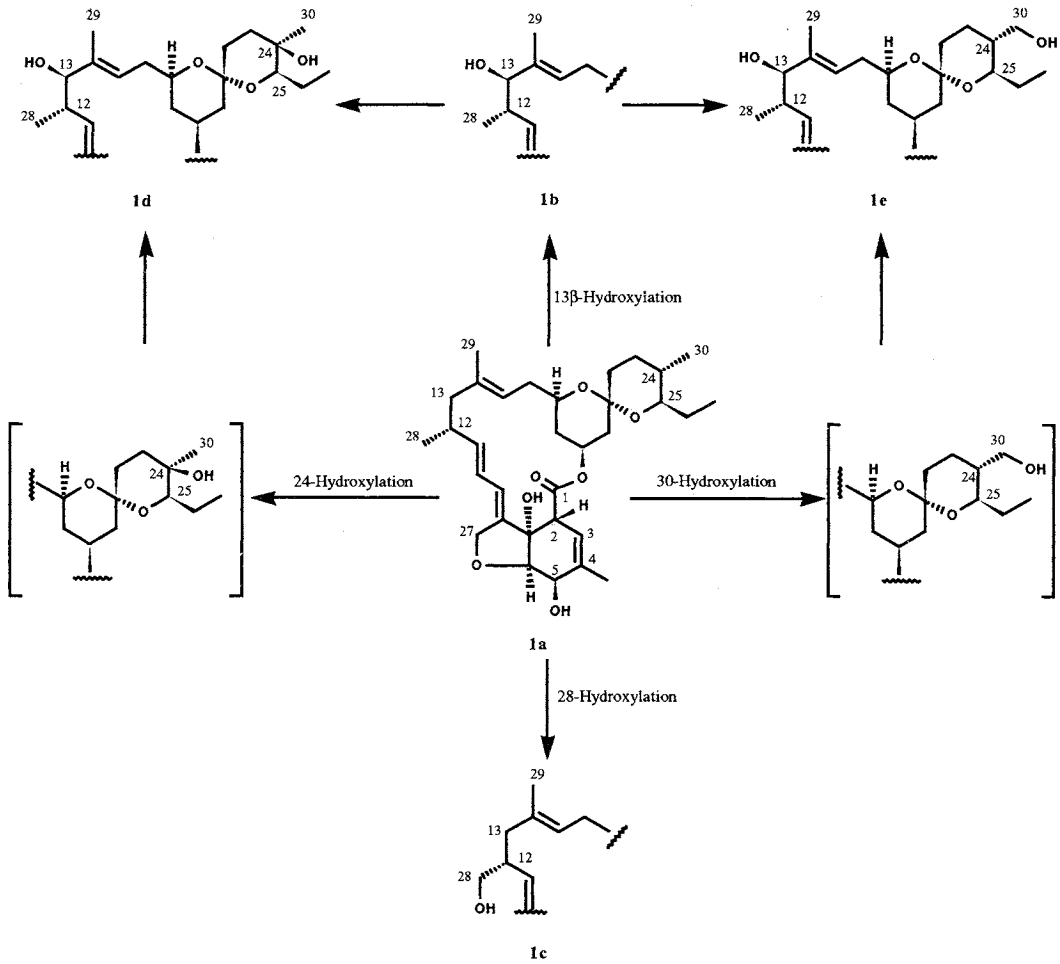
	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
1b	CH_2CH_3	H	H	H	H	OH	H	H	OH
1d	CH_2CH_3	OH	H	H	H	OH	H	H	OH
1e	CH_2CH_3	H	OH	H	H	OH	H	H	OH
2b	CH_3	H	H	H	H	OH	H	H	OH
3b	$CH(CH_3)_2$	H	H	H	H	OH	H	H	OH
3c	$CH(CH_3)_2$	H	H	H	H	H	OH	H	OH
4b	CH_2CH_3	H	H	H	OH	OH	H	H	OH
6b	CH_2CH_3	H	H	H	H	OH	H	NOH	
6d	CH_2CH_3	OH	H	H	H	OH	H	NOH	
6e	CH_2CH_3	H	OH	H	H	OH	H	NOH	
8b	$^{31}C(CH_3)=^{32}CH^{33}CH(CH_3)_2$	H	H	OH	H	OH	H	H	OH
8c	$^{31}C(CH_3)=^{32}CH^{33}CH(CH_3)_2$	H	H	OH	H	H	OH	H	OH

given below. The structures of conversion products are shown in Fig. 2.

Production of the hydroxylated derivatives of milbemycins A_3 (**2a**), A_4 (**1a**), and D (**3a**), were estimated quantitatively as a function of cultivation time. These 13β-hydroxylated derivatives and 28-hydroxymilbemycin D (**3c**) persisted at constant levels from 4 days until the end of culture at 8 days. Among them, 13β-hydroxymilbemycin A_4 (**1b**) was produced most efficiently. Hydroxylation products at the C-24 or C-30 position were also obtained as 13β,24- or 13β,30-dihydroxy compounds from milbemycin A_4 (**1a**) and 5-ketomilbemycin A_4 5-oxime (**6a**). From the time course profile of milbemycin A_4 (**1a**), these compounds were detected 1 day or 2 days following the production of the 13β-hydroxy derivative (**1b**). However, it can not be determined whether these compounds arise from 13β-hydroxymilbemycin A_4 (**1b**) or from the preformed 24- or 30-hydroxymilbemycin A_4 (**1d** or **1e**). Both the 13β- and 28-hydroxy derivatives were obtained from milbemycin D (**3a**) and LL-F28249α (**8a**). A trace amount of 28-hydroxymilbemycin A_4 was also detected by HPLC (data not shown). Variation in the yield of 13β- and 28-hydroxy derivatives suggested that the rather large bulk of the C-25 side chain caused the decrease in the yield of 13β-hydroxy derivatives and the increase in the yield of 28-hydroxy derivatives. The proposed bioconversion pathway of milbemycin A_4 (**1a**) by *C. echinulata* ATCC 9244 is presented in Fig. 3. Compared with milbemycins, the hydroxylated compounds newly obtained in this study did not improve acaricidal activities.

RAMOS TOMBO *et al.* reported 13β-hydroxylation and 14,15-epoxydation of milbemycins by *S. violascens*

Fig. 3. Proposed pathway for bioconversion of milbemycin A_4 by *Cunninghamella echinulata* ATCC 9244.



ATCC 31560[7]. In contrast with *Streptomyces* in their report, *C. echinulata* ATCC 9244 in our study showed C-24, C-28, and C-30 hydroxylation on milbemycins in addition to 13β-hydroxylation, but did not 14,15-epoxydation.

In summation, the research described above establishes efficient microbial hydroxylation of milbemycins and related compounds at the C-13β position by *C. echinulata* ATCC 9244. Further work is under way to thoroughly define the scope of the microbial conversions of milbemycins and related compounds.

Physico-chemical Properties

The ^{1}H NMR spectral data are listed in Table 4.

13β-Hydroxymilbemycin A_4 (**1b**): IR (KBr) cm^{-1} 3600~3200 (br s), 2959 (s), 2915 (s), 2860 (s), 1705 (s), 1612 (w); MS *m/z* 558 (M, $C_{32}H_{46}O_8$), 540, 430, 279, 195, 167; HREI-MS calcd for $C_{32}H_{46}O_8$: 558.3193, found: 558.3194.

13β,24-Dihydroxymilbemycin A_4 (**1d**): IR (KBr) cm^{-1} 3700~3100 (br s), 2969 (s), 2932 (s), 2874 (s), 1718 (s), 1675 (s); MS *m/z* 574 (M, $C_{32}H_{46}O_9$), 556, 386, 295, 183, 167; HREI-MS calcd for $C_{32}H_{46}O_9$:

Table 4. ^{1}H NMR spectral data of conversion products in $CDCl_3$ (270 MHz).

1b	5.75~5.85 (2H, m, 9-H, 10-H), 5.31~5.40 (3H, m, 3-H, 11-H, 19-H), 5.24 (1H, t, J=7.3 Hz, 15-H), 4.70(1H, d, J=15.0 Hz, 27-H), 4.69 (1H, d, J=15.0 Hz, 27-H), 4.30 (1H, br s, 5-H), 4.03 (1H, s, 7-OH), 3.96 (1H, d, J=6.2 Hz, 6-H), 3.72 (1H, d, J=9.9 Hz, 13-H), 3.58 (1H, m, 17-H), 3.27 (1H, q, J=2.2 Hz, 2-H), 3.07 (1H, dt, J_d=2.4 Hz, J_t=9.3 Hz, 25-H), 2.26~2.39 (4H, m, 5-OH, 12-H, 16-H_2), 2.01 (1H, m, 20-H), 1.88 (3H, t, J=1.8 Hz, 26-H_3), 1.59 (3H, s, 29-H_3), 1.48~1.75 (6H, m, 18-H, 22-H_2, 23-H_2, 31-H), 1.25~1.42 (3H, m, 20-H, 24-H, 31-H), 1.13 (3H, d, J=6.6 Hz, 28-H_3), 0.99 (3H, t, J=7.0 Hz, 32-H_3), 0.90 (1H, m, 18-H), 0.83 (3H, d, J=6.2 Hz, 30-H_3)
1d	5.75~5.89 (2H, m, 9-H, 10-H), 5.29~5.43 (3H, m, 3-H, 11-H, 19-H), 5.22 (1H, t, J=7.7 Hz, 15-H), 4.67, 4.73 (2H, ABq, J=14.5 Hz, 27-H_2), 4.29 (1H, t, J=6.2 Hz, 5-H), 3.96 (1H, d, J=6.2 Hz, 6-H), 3.91 (1H, s, 7-OH), 3.72 (1H, d, J=9.9 Hz, 13-H), 3.60 (1H, m, 17-H), 3.33 (1H, dd, J=3.0, 10.3 Hz, 25-H), 3.27 (1H, q, J=2.2 Hz, 2-H), 2.21~2.42 (4H, m, 5-OH, 12-H, 16-H_2), 2.10 (1H, dd, J=3.0, 12.5 Hz, 20-H), 1.88 (3H, s, 26-H_3), 1.58 (3H, s, 29-H_3), 1.20~1.95 (8H, m, 18-H, 20-H, 22-H_2, 23-H_2, 31-H_2), 1.14 (3H, d, J=5.5 Hz, 28-H_3), 1.13 (3H, s, 30-H_3), 1.04 (3H, t, J=7.3 Hz, 32-H_3), 0.80~0.95 (1H, m, 18-H)
2b	5.72~5.91 (2H, m, 9-H, 10-H), 5.21~5.45 (4H, m, 3-H, 11-H, 15-H, 19-H), 4.70 (1H, d, J=15.7 Hz, 27-H), 4.69 (1H, d, J=15.7 Hz, 27-H), 4.29 (1H, d, J=6.1 Hz, 5-H), 4.04 (1H, s, 7-OH), 3.96 (1H, d, J=6.1 Hz, 6-H), 3.71 (1H, d, J=9.6 Hz, 13-H), 3.55 (1H, m, 17-H), 3.20~3.30 (2H, m, 2-H, 25-H), 2.20~2.40 (4H, m, 5-OH, 12-H, 16-H_2), 1.95~2.05 (1H, m, 20-H), 1.87 (3H, s, 26-H_3), 1.58 (3H, s, 29-H_3), 1.20~1.80 (7H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H), 1.15 (3H, d, J=6.1 Hz, 31-H_3), 1.13 (3H, d, J=6.4 Hz, 28-H_3), 0.84 (3H, d, J=6.9 Hz, 30-H_3), 0.8~1.0 (1H, m, 18-H)
3b	5.75~5.85 (2H, m, 9-H, 10-H), 5.30~5.41 (3H, m, 3-H, 11-H, 19-H), 5.23 (1H, m, 15-H), 4.71 (1H, d, J=15.0 Hz, 27-H), 4.70 (1H, d, J=15.0 Hz, 27-H), 4.30 (1H, d, J=6.2 Hz, 5-H), 4.02 (1H, br s, 7-OH), 3.97 (1H, d, J=6.2 Hz, 6-H), 3.72 (1H, d, J=9.4 Hz, 13-H), 3.60 (1H, m, 17-H), 3.27 (1H, q, J=2.2 Hz, 2-H), 3.08 (1H, dd, J=9.2, 1.8 Hz, 25-H), 2.15~2.42 (4H, m, 5-OH, 12-H, 16-H_2), 2.01 (1H, m, 20-H), 1.88 (3H, s, 26-H_3), 1.25~1.75 (11H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 29-H_3, 31-H), 1.13 (3H, d, J=6.6 Hz, 28-H_3), 1.05 (3H, d, J=6.6 Hz, 32-H_3), 0.86 (3H, d, J=6.6 Hz, 33-H_3), 0.80~1.0 (1H, m, 18-H), 0.80 (3H, d, J=5.9 Hz, 30-H_3)
3c	5.80~5.95 (2H, m, 9-H, 10-H), 5.30~5.43 (3H, m, 3-H, 11-H, 19-H), 5.01 (1H, t, J=7.7 Hz, 15-H), 4.66, 4.73 (2H, ABq, J=14.3 Hz, 27-H_2), 4.30 (1H, d, J=6.0 Hz, 5-H), 3.97 (1H, d, J=6.0 Hz, 6-H), 3.60 (1H, m, 17-H), 3.55 (1H, dd, J=5.1, 10.6 Hz, 28-H), 3.39 (1H, dd, J=8.4, 10.6 Hz, 28-H), 3.28 (1H, m, 2-H), 3.08 (1H, dd, J=1.8, 9.2 Hz, 25-H), 2.52 (1H, m, 12-H), 2.2~2.3 (3H, m, 13-H, 16-H_2), 2.00 (1H, dd, J=3.3, 12.1 Hz, 20-H), 1.88 (3H, s, 26-H_3), 1.55 (3H, s, 29-H_3), 1.25~1.95 (9H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H), 1.06 (3H, d, J=6.8 Hz, 32-H_3), 0.86 (3H, d, J=6.8 Hz, 33-H_3), 0.80~0.95 (1H, m, 18-H), 0.80 (3H, d, J=5.9 Hz, 30-H_3)
4b	5.72~5.90 (2H, m, 9-H, 10-H), 5.25~5.45 (4H, m, 3-H, 11-H, 15-H, 19-H), 4.66, 4.71 (2H, ABq, J=14.5 Hz, 27-H_2), 4.13, 4.47 (2H, ABq, J=12.5 Hz, 29-H_2), 4.29 (1H, d, J=6.4 Hz, 5-H), 4.03 (1H, s, 7-OH), 3.96 (1H, d, J=6.4 Hz, 6-H), 3.78 (1H, d, J=10.1 Hz, 13-H), 3.59 (1H, m, 17-H), 3.27 (1H, m, 2-H), 3.07 (1H, dt, J_d=2.4 Hz, J_t=9.0 Hz, 25-H), 2.55 (1H, m, 12-H), 2.21~2.42 (3H, m, 5-OH, 16-H_2), 1.99 (1H, dd, J=3.0, 12.1 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.20~1.80 (9H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.18 (3H, d, J=6.5 Hz, 28-H_3), 0.99 (3H, t, J=7.5 Hz, 32-H_3), 0.83 (3H, d, J=6.5 Hz, 30-H_3), 0.75~0.90 (1H, m, 18-H)
6b	8.17 (1H, br s, 5=NOH), 5.70~5.89 (3H, m, 3-H, 9-H, 10-H), 5.30~5.48 (2H, m, 11-H, 19-H), 5.23 (1H, t, J=7.9 Hz, 15-H), 4.76 (1H, d, J=14.7 Hz, 27-H), 4.71 (1H, d, J=14.7 Hz, 27-H), 4.67 (1H, s, 6-H), 3.95 (1H, s, 7-OH), 3.73 (1H, d, J=9.7 Hz, 13-H), 3.60 (1H, m, 17-H), 3.38 (1H, m, 2-H), 3.08 (1H, dt, J_d=2.2 Hz, J_t=9.2 Hz, 25-H), 2.20~2.42 (3H, m, 12-H, 16-H_2), 2.02 (1H, m, 20-H), 1.93 (3H, t, J=1.1 Hz, 26-H_3), 1.58 (3H, s, 29-H_3), 1.20~1.80 (9H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.14 (3H, d, J=6.6 Hz, 28-H_3), 0.99 (3H, t, J=7.3 Hz, 32-H_3), 0.90 (1H, m, 18-H), 0.83 (3H, t, J=6.6 Hz, 30-H_3)
6d	8.48 (1H, br s, 5=NOH), 5.74~5.88 (3H, m, 3-H, 9-H, 10-H), 5.34~5.44 (2H, m, 11-H, 19-H), 5.22 (1H, m, 15-H), 4.76 (1H, d, J=14.3 Hz, 27-H), 4.72 (1H, d, J=14.3 Hz, 27-H), 4.67 (1H, s, 6-H), 3.85 (1H, s, 7-OH), 3.73 (1H, d, J=9.5 Hz, 13-H), 3.60 (1H, m, 17-H), 3.39 (1H, t, J=2.2 Hz, 2-H), 3.34 (1H, dd, J=9.9, 2.9 Hz, 25-H), 2.26~2.43 (3H, m, 12-H, 16-H_2), 2.10 (1H, dd, J=11.7, 4.4 Hz, 20-H), 1.94 (3H, s, 26-H_3), 1.59 (3H, s, 29-H_3), 1.52~1.89 (7H, m, 18-H, 22-H_2, 23-H_2, 31-H_2), 1.40 (1H, t, J=11.7 Hz, 20-H), 1.14 (3H, d, J=5.1 Hz, 28-H_3), 1.13 (3H, s, 30-H_3), 1.04 (3H, t, J=7.3 Hz, 32-H_3), 0.82~1.1 (1H, m, 18-H)
6e	8.02 (1H, br s, 5=NOH), 5.76~5.88 (3H, m, 3-H, 9-H, 10-H), 5.33~5.46 (2H, m, 11-H, 19-H), 5.23 (1H, t, J=7.7 Hz, 15-H), 4.77 (1H, d, J=14.3 Hz, 27-H), 4.72 (1H, d, J=14.3 Hz, 27-H), 4.67 (1H, s, 6-H), 3.90 (1H, s, 7-OH), 3.72 (1H, d, J=9.9 Hz, 13-H), 3.64 (1H, dd, J=11.0, 3.7 Hz,

Table 4. (Continued)

	30-H), 3.52 (1H, dd, J=11.0, 6.2 Hz, 30-H), 3.48~3.67 (1H, m, 17-H), 3.38 (1H, t, J=2.2 Hz, 2-H), 3.31~3.39 (1H, m, 25-H), 2.26~2.43 (3H, m, 12-H, 16-H_2), 2.03 (1H, dd, J=12.1, 3.7 Hz, 20-H), 1.94 (3H, q, J=1.5 Hz, 26-H_3), 1.59 (3H, s, 29-H_3), 1.25~1.79 (9H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.14 (3H, d, J=6.6 Hz, 28-H_3), 1.02 (3H, t, J=7.0 Hz, 32-H_3), 0.86~1.05 (1H, m, 18-H)
8b	5.70~5.88 (2H, m, 9-H, 10-H), 5.20~5.45 (4H, m, 3-H, 11-H, 19-H, 32-H), 5.13 (1H, m, 15-H), 4.69 (2H, ABq, J=14.3 Hz, 27-H_2), 4.28 (1H, br s, 5-H), 3.95 (1H, d, J=6.0 Hz, 6-H), 3.83 (1H, s, 7-OH), 3.78 (1H, br s, 23-H), 3.75 (1H, d, J=11.8 Hz, 25-H), 3.71 (1H, d, J=9.7 Hz, 13-H), 3.50~3.75 (2H, m, 17-H, 23-OH), 3.26 (1H, m, 2-H), 2.80 (1H, m, 12-H), 2.60 (1H, m, 33-H), 1.95~2.40 (5H, m, 5-OH, 16-H_2, 20-H, 22-H), 1.87 (3H, s, 26-H_3), 1.61 (3H, s, 31-CH_3), 1.59 (3H, s, 29-H_3), 1.20~1.85 (4H, m, 18-H, 20-H, 22-H, 24-H), 1.13 (3H, d, J=6.5 Hz, 28-H_3), 1.05 (3H, d, J=6.4 Hz) & 0.96 (3H, d, J=6.4 Hz) (33-$(CH_3)_2$), 0.85~0.95 (1H, m, 18-H), 0.80 (3H, d, J=6.9 Hz, 30-H_3)
8c	5.81~5.94 (2H, m, 9-H, 10-H), 5.41 (1H, s, 3-H), 5.26~5.42 (2H, m, 11-H, 19-H), 5.20 (1H, dd, J=8.9, 1.2 Hz, 32-H), 5.01 (1H, m, 15-H), 4.71 (1H, dd, J=14.7, 2.0 Hz, 27-H), 4.70 (1H, dd, J= 14.7, 2.0 Hz, 27-H), 4.28 (1H, br s, 5-H), 3.95 (1H, d, J=6.5 Hz, 6-H), 3.91 (1H, s, 7-OH), 3.80 (1H, m, 23-H), 3.75 (1H, d, J=10.9 Hz, 25-H), 3.52~3.70 (3H, m, 17-H, 23-OH, 28-H), 3.39 (1H, dd, J=10.5, 8.1 Hz, 28-H), 3.27 (1H, q, J=2.4 Hz, 2-H), 2.45~2.62 (2H, m, 12-H, 33-H), 2.17~2.37 (4H, 5-OH, 13-H, 16-H_2), 1.94~2.09 (2H, m, 20-H, 22-H), 1.87 (3H, s, 26-H_3), 1.61 (3H, d, J=1.2 Hz, 31-CH_3), 1.56 (3H, s, 29-H_3), 1.20~1.90 (5H, m, 13-H, 18-H, 20-H, 22-H, 24-H), 1.05 (3H, d, J=6.4 Hz, 33-CH_3), 0.95 (3H, d, J=6.4 Hz, 33-CH_3), 0.80 (3H, d, J=6.8 Hz, 30-H_3), 0.80~1.00 (1H, m, 18-H)

δ ppm downfield from internal TMS.

574.3142, found: 574.3134.

13β-Hydroxymilbemycin A_3 (**2b**): IR (KBr) cm^{-1} 3700~3100 (br s), 2960 (s), 2920 (s), 2869 (s), 1750 (s); MS m/z 544 (M, $C_{31}H_{44}O_8$), 526, 508, 265, 181, 153, 129; HREI-MS calcd for $C_{31}H_{44}O_8$: 544.3036, found: 544.3030.

13β-Hydroxymilbemycin D (**3b**): IR (KBr) cm^{-1} 3600~3200 (br s), 2960 (s), 2930 (s), 2870 (m), 2860 (m), 1714 (s), 1620 (w); MS m/z 572 (M, $C_{33}H_{48}O_8$), 554, 444, 426, 293, 209, 181, 151; HREI-MS calcd for $C_{33}H_{48}O_8$: 572.3349, found: 572.3358.

28-Hydroxymilbemycin D (**3c**): IR (KBr) cm^{-1} 3700~3100 (br s), 2961 (s), 2928 (s), 2872 (s), 2859 (s), 1714 (s); MS m/z 572 (M, $C_{33}H_{48}O_8$), 444, 426, 372, 330, 278, 259, 209, 181, 167; HREI-MS calcd for $C_{33}H_{48}O_8$: 572.3349, found: 572.3352.

13β,29-Dihydroxymilbemycin A_4 (**4b**): IR (KBr) cm^{-1} 3700~3100 (br s), 2963 (s), 2930 (s), 2873 (s), 1717 (s); MS m/z 556 (M$-H_2O$, $C_{32}H_{44}O_8$), 295, 279, 237, 195, 167, 151; HREI-MS calcd for $C_{32}H_{46}O_9$: 574.3142, found: 574.3149; $C_{32}H_{44}O_8$: 556.3036, found: 556.3044.

13β-Hydroxy-5-ketomilbemycin A_4 5-oxime (**6b**): IR (KBr) cm^{-1} 3700~2900 (br s), 2960 (s), 2930 (s), 2870 (s), 1750 (s); MS m/z 571 (M, $C_{32}H_{45}NO_8$), 553, 279, 195, 167; HREI-MS calcd for $C_{32}H_{45}NO_8$: 571.3146, found: 571.3148.

13β,24-Dihydroxy-5-ketomilbemycin A_4 5-oxime (**6d**): IR (KBr) cm^{-1} 3600~3200 (br s), 2971 (s), 2930 (s), 2874 (s), 1716 (s), 1680 (w); MS m/z 587 (M, $C_{32}H_{45}NO_9$), 569, 553, 295, 211, 183; HREI-MS calcd for $C_{32}H_{45}NO_9$: 587.3094, found: 587.3101.

13β,30-Dihydroxy-5-ketomilbemycin A_4 5-oxime (**6e**): IR (KBr) cm^{-1} 3600~3100 (br s), 2958 (s), 2926 (s), 2874 (s), 1722 (s), 1668 (s); MS m/z 587 (M, $C_{32}H_{45}NO_9$), 569, 553, 295, 211, 183; HREI-MS calcd for $C_{32}H_{45}NO_9$: 587.3094, found: 587.3050.

13β-Hydroxy LL-F28249α (**8b**): IR (KBr) cm^{-1} 3650~3100 (br s), 2959 (s), 2928 (s), 2869 (s), 1719 (s), 1674 (m); MS m/z 628 (M, $C_{36}H_{52}O_9$), 612, 610, 368, 264, 236; HREI-MS calcd for $C_{36}H_{52}O_9$:

628.3611, found: 628.3635.

28-Hydroxy LL-F28249α (**8c**): IR (KBr) cm^{-1} 3600~3200 (br s), 2959 (s), 2927 (s), 2869 (s), 1719 (s); MS *m/z* 628 (m, $C_{36}H_{52}O_9$), 610, 592, 482, 330, 167, 151; HREI-MS calcd for $C_{36}H_{52}O_9$: 628.3611, found: 628.3600.

References

1) TAKIGUCHI, Y.; H. MISHIMA, M. OKUDA, M. TERAO, A. AOKI & R. FUKUDA: Milbemycins, a new family of macrolide antibiotics: Fermentation, isolation and physico-chemical properties. J. Antibiotics 33: 1120~1127, 1980
2) OKAZAKI, T.; M. ONO, A. AOKI & R. FUKUDA: Milbemycins, a new family of macrolide antibiotics: Producing organism and its mutants. J. Antibiotics 36: 438~441, 1983
3) MISHIMA, H.; J. IDE, S. MURAMATSU & M. ONO: Milbemycins, a new family of macrolide antibiotics. Structure determination of milbemycins D, E, F, G, H, J and K. J. Antibiotics 36: 980~990, 1983
4) ALBERS-SCHÖNBERG, G.; B. H. ARISON, J. C. CHABALA, A. W. DOUGLAS, P. ESKOLA, M. H. FISHER, A. LUSI, H. MROZIK, J. L. SMITH & R. L. TOLMAN: Avermectins. Structure determination. J. Am. Chem. Soc. 103: 4216~4221, 1981
5) CARTER, G. T.; J. A. NIETSCHE, M. R. HERTZ, D. R. WILLIAMS, M. M. SIEGEL, G. O. MORTON, J. C. JAMES & D. B. BORDERS: LL-F28249 antibiotic complex: A new family of antiparasitic macrocyclic lactones. Isolation, characterization and structures of LL-F28249 α, β, γ, λ. J. Antibiotics 41: 519~529, 1988
6) NAKAGAWA, K.; A. TORIKATA, K. SATO & Y. TSUKAMOTO: Microbial conversion of milbemycins: 30-Oxidation of milbemycin A_4 and related compounds by *Amycolata autotrophica* and *Amycolatopsis mediterranei*. J. Antibiotics 43: 1321~1328, 1990
7) RAMOS TOMBO, G. M.; O. GHISALBA, H.-P. SCHÄR, B. FREI, P. MAIENFISCH & A. C. O'SULLIVAN: Diastereoselective microbial hydroxylation of milbemycin derivatives. Agric. Biol. Chem. 53: 1531~1535, 1989
8) GEHRET, J. C. (Ciba-Geigy A.-G.): Preparation of 29-(carbonyloxy)milbemycins for controlling parasites in animals and plants. Eur. Pat. Appl. 237 482, Mar. 6, 1986 [CA 110: 134974u, 1989]
9) IDE, J.; S. MURAMATSU, Y. NAKADA & N. KITANO (Sankyo): Didehydromilbemycin derivatives. Eur. Pat. Appl. 110 667, Nov. 25, 1982 [CA 101: 210854m, 1984]
10) SATO, K.; T. YANAI, T. KINOTO & S. MIO (Sankyo): Milbemycin derivatives and compounds for their preparation. Eur. Pat. Appl. 184 308, Oct. 26, 1984 [CA 106: 67005f, 1987]